APPLICATION

FOR

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TITLE: MULTIPLEXED STRAND DISPLACEMENT FOR NUCLEIC ACID DETERMINATIONS

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CROSS REFERENCE TO RELATED APPLICATIONS

This application is a Continuation-In-Part of U.S. Serial Number 09/354,629, filed on July 16, 1999, which disclosure is hereby incorporated by reference.

INTRODUCTION

Background

Knowledge of nucleic acid sequences of species is increasingly important for many different purposes. The large number of bases in even the simplest genomes makes the sequencing of the genomes for the different species of interest a daunting task. Not only is there interest in a consensus sequence for a particular species, but there is also interest in determining the differences between individuals of a single species. There are many motivations for sequencing nucleic acids, including de novo sequencing, or resequencing for comparative, confirmatory or forensic applications and the like. In order to improve the rate at which nucleic acid sequences can be determined, automated machines have been devised, which primarily rely on the use of the polymerase chain reaction to amplify genomic DNA fragments, followed by primer extension and termination, using capillary electrophoresis for analysis. Using a single primer for the primer extension is slow, and inefficient in terms of throughput and reagent usage. Desirably, one would wish to have a plurality of different primers, e.g. multiplexed primers, spaced along a long strand of DNA or different strands of DNA, so that one could simultaneously sequence multiple kilobases. Multiplexing of this type is problematic without a method to separate the extended fragments from each of the regions, in order to be able to define the sequences.

In addition to sequencing there are many types of genetic analysis methods for which the means to multiplex would be of great benefit. For example, the possibility of using groups of single nucleotide polymorphisms ("SNPs") to characterize or identify different traits has

become an area of major interest. This area of interest is presently in the phase of cataloging SNPs. Once a sufficient number of SNPs have been identified for a statistically significant population, the major effort of relating SNP profiles to traits will begin. In each instance there will be a large number of nucleotide determinations to be made by, for example, single base primer extension or ligation reactions, which, if made individually will be very inefficient and expensive. Multiplexing provides greater efficiencies of throughput and cost because many reactions are run simultaneously in the same pool of reagents. However, the efforts to use multiplexing are confounded by the large number of DNA, RNA, or nucleotide molecules involved, the errors that inherently occur and the possibility of their amplification, and the impediments to separation of the sequences to obtain substantially pure fractions. Also, there is the desirability, to the extent it is possible, to recover and reuse reagents.

Although many methods for sequence-specific or primer-specific DNA purification methods have been described, including methods based on triplex affinity capture, peptide nucleic acid mediated capture, sequence affinity, or biotin capture, they do not address the means to perform the purification of multiplexed samples with independent release of the multiplex components.

There is, therefore, substantial interest in developing improved processes for performing genetic analyses using multiplexed protocols that provide for accuracy, efficiency in the use of time, equipment and reagents and reproducibility.

Brief Description of the Prior Art

U.S. Patent No. 5,648,213 discloses the use of strand displacement. U.S. Patent nos. 5,514,543 and 5,580,732 describe DNA sequence detection using multiple probes in a single assay. WO98/US000207 describes biopolymers attached to a support with a reversible link. WO98/14610 describes multiplex polynucleotide capture methods and compositions. EP 0 416 817 describes primers containing polynucleotide tails.

SUMMARY OF THE INVENTION

Methods and compositions are provided for multiplexed determinations of at least one characteristic of a plurality of target moieties. In a single step, the plurality of moieties is processed to provide an assemblage of assay entities to be defined to provide the characteristic(s) of interest of the target moieties. The method is exemplified with nucleic acids as the target moieties, for sequencing, genotyping, and the like. By using a plurality of different primers, modifying the primers, sequestering the (modified) primers, releasing the (modified) primers independently using selective strand displacement, and assaying the released (modified) primers, multiplexed determinations are performed for identifying at least one characteristic of a target moiety. The compositions employed comprise sequestering supports having a plurality of capture probes for the capture and subsequent selective release of a primer probe in combination with strand displacement probes. Also present may be labels or identifiers, which further allow for differentiation of the different primers.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure **1** shows a multiplexed set of primers differentiated by identifier and capture sequence.

Figure **2** shows the electropherogram of a four-plex mixture released simultaneously by strand displacement.

Figures **3A – 3D** show the consecutive electropherograms of the same four-plex mixture released independently by strand displacement.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and compositions are disclosed for multiplexed determinations of target moieties. The methods and compositions employ nucleic acid sequences as primers, as capture probes for the capture of homologous nucleic acid sequences (e.g. primers), and as strand displacers for the selective release of the primers from the capture probes. In performing the method, a mixture of primers specific for target nucleic acid sequences are combined with single-stranded target moieties under hybridization conditions, wherein primer/target duplexes are formed. The primers are then modified in various ways, depending

on the characteristic of interest of the nucleic acid target moiety. The modified primers are released from the target moieties and captured by homologous nucleic acid sequences (capture probes) for sequestering the modified primers. The modified primers are optionally washed to remove unbound components, and are then independently released from the support by sequentially adding strand displacement probes, each specific for one of the capture probes and having a greater binding affinity for the capture probe than do the primers. The primers are released from the capture probes as the strand displacement probes replace the primers from their common binding region. Each group of released extended primers may then be assayed for the characteristic of interest.

The subject methods comprise identification of target species in a complex mixture, where a large number of different target species are of interest. The subject method finds particular application for nucleic acid sequencing, single nucleotide polymorphism ("SNP") determinations, fragment identification, genotyping in association with cell strains, phenotypes, etc., allelic profiling and the like. Normally, these determinations are made in the presence of a large amount of nucleic acid, such as a cellular genome, cDNA transcripts from a cell, a complex mixture of DNA and RNA, and the like. Generally, the amount of DNA or RNA will be at least about 2 kb, more usually at least about 5 kb, and may be a full genomic complement. Within this amount of DNA or RNA only a fraction may be of interest, ranging from around one hundred per trillion bases in the case of SNP typing of human genomes, to up to around 50% in the case of sequencing plasmid inserts.

The reagents required are primer oligonucleotides, capture probe oligonucleotides, which are bound to or capable of being bound to a support, and strand displacer oligonucleotides. Each of these oligonucleotides will usually differ as to composition and function.

In referring to oligonucleotides, it is intended to include not only the naturally occurring nucleotides, but nucleotides which are functionally equivalent for the purposes of this invention. The nucleotides may be varied as to the backbone, the phosphate and sugar being replaced with equivalent moieties, such as phosphoramides, amino acids, phosphotriesters, methyl phosphonates, thiophosphates, thiophosphoramides, arabinosides,

etc. Both natural and unnatural bases and sugars may be employed that provide the desired binding affinity with a homologous sequence.

For example, a number of unnatural bases are found to have higher binding affinity than the natural bases they replace, such as, for example 5-(1-propynyl)uracil, and 5-(1-propynyl)cytosine, which are described in U.S. Patent No. 5,830,653. Also, some unnatural nucleotide structures have been shown to have higher binding affinity to nucleic acid sequences than a natural nucleotide structure, particularly peptide nucleic acids (PNA), where the phosphate ester backbone is substituted with a polyamide backbone (Nielsen, P.E., Egholm, M., Berg, R.H. and Buchardt, O. "Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide" *Science 254* (1991) 1497-1500; Nielsen, P.E. et al., U.S. Patent No. 5,539,082; Buchardt, O., Egholm, M., Nielsen, P.E. and Berg, R.H. "Peptide Nucleic Acids" *PCT WO 92/20702* (1992); Thomson, S., Noble, S. and Ricca, D. "Peptide nucleic acids and the effect on genetic material" *PCT Appl. WO 93/12129* (1993)). In particular, it may be advantageous to enhance the activity of the strand displacement probe, wherein the probe comprises unnatural components that increase the binding affinity of the probe. Also, it may be advantageous to pairwise substitute bases in any pair of probes that are to bind to one another with moieties that preferentially bind to each other rather than to any of the natural bases. For example, iso-dC and iso-dG bind to one another but will not effectively base pair with adenosine, thymidine, cytidine, guanosine, or uridine, as described in U.S. Patent No. 5,681,702.

The primer oligonucleotide will preferably have two nucleic acid sequence parts, and optionally a non-replicable moiety between, or a junction preventing polymerase activity across the two nucleic acid sequence parts. Primers of this general type have been described earlier, in EP 416817 and WO 94/21820, which are herein incorporated by reference. The first sequence part is a target binding sequence comprising at least about 8 nucleotides, more usually at least about 12 nucleotides and which may have 30 nucleotides or more. The greater the number of complementary nucleotides, the greater the specificity is for the target. The greater the complexity of the target composition, the more desirable it is to have a longer oligonucleotide. The primer oligonucleotide will optionally have a second sequence part designed to hybridize to the capture oligonucleotide, having at least 5 nucleotides, usually at

least 6 nucleotides and may have as many as 30 or more, more usually not more than about 20 nucleotides. The considerations are the desired level of specificity, the costs of preparing the oligonucleotides, the level of complexity of the sample composition, and the like. Usually, the oligonucleotide portions together will be at least 13 nucleotides, more usually at least about 18 nucleotides, and not more than about 50 nucleotides.

The optional non-replicable moiety linking the two sequence parts of the primer oligonucleotide is comprised of at least two atoms in the chain and not more than about 120 atoms in the chain, preferably not more than about 60 atoms in the chain, more preferably not more than about 30 atoms in the chain. A methylene chain may be used, such as propyl, dodecyl, octadecyl and the like. Where solubility is a consideration, the chain may also contain one or more heteroatoms, usually selected from nitrogen, oxygen, sulfur and phosphorus, although other atoms may also be present. Conveniently, the linking group may be an amino acid or polypeptide, a polyether such as polyoxyethylene, non-replicable nucleotides, a polyester, etc. Polyoxyethylenes such as di-, tri-, tetra-, penta-, hexaethyleneglycol and the like are particularly useful for their conformational flexibility and hydrophilicity. Generally, the linking group will be coupled to the oligonucleotide sequences via phosphodiester groups and the like, using standard coupling chemistry compatible with automated synthesizers.

Where the primer oligonucleotide is to be used in a polymerase chain reaction, a means to prevent the polymerase from extending into the capture sequence part of the oligonucleotide and rendering that part double stranded is desirable. A non-nucleosidic linking group incorporated between the two sequence parts is desirable for its ability to act as a PCR stop. The polymerase enzyme will not extend across the link because it is non-nucleosidic. Alternatively, a means to create a PCR stop without incorporating a linking group is to prepare the two contiguous sequence parts with reversed 5'– 3' orientation of the two sequences. In other applications, the linking group may be desirable for providing steric relief for the different hybridizing components.

The first sequence part will be substantially homologous, usually fully complementary to the target sequence. Usually, there will be less than 20% homology difference, more usually less than about 15% homology difference between the oligonucleotide sequence and

the target sequence, as determined by such methods as described in Shpaer et al., Genomics 1996, 38:179-91; States and Agarwal, Ismb 1996; 4:211-7; Mott et al., Comput Appl Biosci 1989, 5:123-31; and Pearson and Lipman, PNAS USA 1988, 85:2444-8, particularly the LFASTA method. For the most part there will be fewer than 5, more usually fewer than 3, preferably not more than about 1, nucleotide substitution, insertion or deletion, or combination thereof, between the first sequence part of the primer oligonucleotide and the target sequence.

The second sequence part, while desirable, is not essential where the first sequence part is unique within the plurality of primer oligonucleotides, as may occur with some sequencing applications. Thus, when sequencing using unique primer sites, the primer oligonucleotide need only include the sequence homologous to the target sequence, where the same sequence will serve to hybridize to the capture oligonucleotide.

Either the first sequence part, as described immediately above, or the second sequence part of the primer oligonucleotide will generally be complementary to the capture oligonucleotide. Usually it will be desirable to have high affinity and specificity in the capture process. Generally, the portions of the primer oligonucleotide and capture oligonucleotide that bind to one another will be complementary and the sequence will be selected to provide unique binding in relation to other sequences which may be present during the capture stage.

However, there may be situations where one wishes to have a single capture nucleic acid generally not more than about 5 kb, usually not more than about 2 kb, yet where differential release may still be obtained. In this situation, one could synthesize a single capture nucleic acid and several primer oligonucleotides, each of which has a second part for binding to different regions of the capture nucleic acid. By appropriately spacing capture sequences complementary to the second part of each primer oligonucleotide on the capture nucleic acid, one could employ a set of primer oligonucleotides that are capable of binding to the same capture nucleic acid but to different regions such that the desired level of independent release is possible by strand displacement. Generally one would wish to have fewer than about 10 different primers captured by a single capture nucleic acid, frequently fewer than 5 different primers, although depending on various considerations such as economics, specificity, sample size and complexity, one could have a single capture nucleic acid for more than 10 primer oligonucleotides.

The capture oligonucleotide is comprised of a capture sequence(s), which binds to the second part of the primer oligonucleotide, and may have a displacement initiation sequence(s). Generally, the capture oligonucleotide will be at least about 8 nucleotides and not more than about 60 nucleotides, usually not more than about 36 nucleotides. A capture sequence of the capture oligonucleotide may be the same length as the second part of the primer oligonucleotide, although in some instances it may be different, usually not more than about 5 bases different.

The capture oligonucleotide will usually include a displacement initiation sequence(s), which will not hybridize with either the primer oligonucleotide or the target sequence. A displacement initiation sequence provides a region for the strand displacement oligonucleotide to bind and promote its invasion into the capture probe/primer duplex, ultimately leading to the displacement of the primer oligo into solution.

The displacement initiation sequence may be contiguous to the capture sequence or separated from it by about 1 – 5, usually 1 – 3, nucleotides, or a non-nucleosidic linking moiety may separate the two sequences. This linking group has a similar composition to the linker optionally included in the primer oligonucleotide, which is described above.

The displacement initiation sequence may be common or differ amongst the different capture oligonucleotides. It may be a homooligo, e.g. poly-dT, etc., or may have a multiplicity of nucleotides. It should be understood that for the purpose of displacement initiation, the diversity of this part of the capture oligonucleotide could be substantially lower than the diversity of the capture sequence. The initiation sequence may be as few as 2 nucleotides, more usually at least about 3 nucleotides and will generally be fewer than 20 nucleotides, usually not more than about 12 nucleotides. The displacement initiation sequence may be 5' or 3' of the capture sequence, and it may be proximal or distal to the capture sequence with respect to the solid support.

In situations where one wishes to have a single capture nucleic acid, a plurality of capture sequences for binding the primer oligonucleotide may be interspersed with displacement initiation sequences. By providing such displacement initiation sequences for each capture sequence, one maintains the ability to independently, and randomly induce the release of each captured primer oligonucleotide.

The capture oligonucleotide may also comprise an ionic moiety or moieties. The moieties may be incorporated as modified nucleotides, or as non-nucleosidic components conjugated to internal or terminal bases, sugars or phosphate groups. Particularly useful are polycationic moieties, such as poly-(amino acids), such as poly-lysine, which may be conveniently coupled to the oligonucleotide, with appropriate modifications via disulfide bond formation or a sulfhydryl-maleimide coupling reaction. Methods for preparing oligonucleotide conjugates are well known. Or, a nucleoside derivative such as an amine-bearing derivative of thymidine may be conveniently incorporated using standard oligonucleotide synthetic methods. The ionic moieties may function to increase the local ionic strength around the hybrids formed in carrying out the subject invention, allowing the concentration of salts in the various buffers to be reduced without adversely affecting the duplex binding affinities and the ability to selectively release the bound multiplex of primers.

The capture oligonucleotide will be bound either directly or indirectly to a support. Supports may include container walls, disks, porous or solid beads, strings, capillary surfaces, polymers, dendritic materials and the like, in effect, any entity which allows for physical separation of what is bound to the entity and what is unbound, and also allows for washing to remove non-specifically bound compounds while retaining specifically bound compounds. Conveniently, the support may be a bead or a spatially defined region of a container, well, or channel.

Usually, a linking group will be employed between the capture oligonucleotide and the support, generally a hydrophilic linking group, conveniently of at least two atoms in the chain and not more than about 120 atoms in the chain, preferably not more than about 60 atoms in the chain, more preferably not more than about 30 atoms in the chain. A methylene chain may be used, such as propyl, dodecyl, octadecyl and the like. Where solubility is a consideration, the chain may also contain one or more heteroatoms, usually selected from nitrogen, oxygen, sulfur and phosphorus, although other atoms may also be present. Conveniently, the linking group may be an amino acid or polypeptide, a polyether such as polyoxyethylene, an ester or a polyester, such as polyglycolide, etc. Polyoxyethylenes such as di-, tri-, tetra-, penta-, hexaethyleneglycol and the like are particularly useful for their conformational flexibility and hydrophilicity.

The chemistry for binding an oligonucleotide to a solid support is well known. The capture oligonucleotide or the linking group may be provided with a chemical reactant or one member of a binding pair suitable for reaction with or binding to an appropriately functionalized support. These methods are known in the art, and include for example biotin/streptavidin binding, or thiol/maleimide adduct formation.

The capture oligonucleotide will typically be bound to the support prior to contact with the sample solution containing the primer oligonucleotide. However, depending on the type of binding chemistry used, it may be convenient for the capture oligonucleotide and the primer oligonucleotide to be combined first, under hybridization conditions, to permit duplex formation. Following this, the duplex is contacted with the support where the capture oligonucleotide, appropriately modified as described above, reacts with or binds to an appropriately functionalized support. The order of combining the reagents may be varied accordingly, such that one forms a structure in which the primer oligonucleotide is reversibly bound to a support via the capture oligonucleotide.

A bead support may be of any convenient composition, such as latex, metal sol, colloidal carbon, polyacrylamide, etc., generally of a diameter in the range of about 1 μ to 1 mm, usually at least about 10 μ, more usually in the range of about 50 μ to 500 μ. The beads may be non-magnetic, diamagnetic or superparamagnetic, depending upon the mode of separation desired. A wide variety of beads are commercially available from different sources. The beads may be functionalized for linking the capture oligonucleotide or may have reactive functionalities for bonding the linking group. If the support is a bead, string, membrane or soluble polymer, it may be further linked to a solid surface such as a container wall, a larger bead or an insoluble polymer to facilitate the manipulation of said support. See, for example, U.S. Patent No. 5,900,481, and references cited therein for a description of beads and conjugation of nucleotides to the beads

The support may be a surface, which may be of any convenient composition, such as plastic, glass, silica, which in turn may be coated with polymers, biopolymers, or other molecules. The coating functions to reduce non-specific adsorption of the analyte or contaminants introduced by the sample itself. The coating may also function in the immobilization of the capture oligonucleotide to the support by providing a chemical reactant

or one member of a binding pair with which the capture oligonucleotide may react. The coating may also comprise polyionic compounds, particularly polycations, such as polylysine or aminated dextrans, which may be included for regulating the ionic strength around the oligonucleotides. The support may also be a porous surface, such as a membrane, e.g. nitrocellulose.

The number of oligonucleotides bonded to an individual support will usually be at least about 10, preferably at least about 50 and may be 10^8 or more, depending on the number of different beads or polymer supports necessary for the assay, the redundancy permitted, the multiplicity of targets, the sensitivity with which the different labels may be distinguished, and the like.

The third oligonucleotide is the strand displacer oligonucleotide. The strand displacer may be comprised of a sequence homologous to the capture oligonucleotide, so as to displace the primer oligonucleotide into solution. The strand displacer may have all or a major portion of the sequence complementary to the capture sequence, generally having at least about 75% of the sequence as contiguous bases. As indicated previously, the strand displacer need not have a natural DNA oligonucleotide composition, but may be substituted in part or in whole depending on the nature of the modification, with other backbones, bases or ionic moieties, particularly those which provide for higher affinity hybridization. Most conveniently, the strand displacer will be complementary to the capture oligonucleotide, although it may vary, especially where the displacement initiation sequence and the capture sequence are not contiguous, or other special situation.

Generally, when complementary, the length of the strand displacement oligonucleotide will equal the length of the capture oligonucleotide. However, the length may be shorter than the length of the capture oligonucleotide as long as the binding affinity is greater than that of the primer oligonucleotide. Where the capture oligonucleotide contains a displacement initiation sequence, a portion of the strand displacement oligonucleotide will hybridize to that sequence. Because the purpose of this hybridization is to promote the strand displacement process, the binding affinity as measured by the T_m should be at least the same as the operating temperature, preferably 5 to 15°C greater than the operating temperature of the assay, and may be 25°C or more above the operating temperature. It should be remembered that T_m will be

affected by the composition of the solution, such as salt concentrations, organic co-solvents, and the like as is well known in the art.

In addition, one may include with the primer oligonucleotide an additional region, which is referred to as an identifier, which during the primer modification process is present and remains part of the modified primer. Depending on the nature of the characterization, the identifier may serve to identify the presence of a particular nucleotide, the composition or identity of the primer, or provide other information of interest about the target nucleic acid. For example, in sequencing and SNP determinations, one may be interested in identifying the particular nucleotide at the terminus of the extended primer without having to uniquely label the terminating nucleotide. More particularly, when performing SNP determinations, i.e. genotyping of individual positions, by single base primer extension using methods such as those described in U.S. Patent No.'s 4,656,127 and 5,888,819, the identifier may be a variable length of nucleotides not integral to the capture sequence or primer sequence portion of the oligonucleotide. Each different primer would be associated with a different identifier. To further increase the capability to multiplex, a similar series of variable length identifiers may be associated with different capture sequences.

In order to avoid sequencing the primer or where variation in mobility of different primers does not provide a sufficiently discrete opportunity for differentiation of the number of target sequences, the identifier may provide for a detectable label on the primer sequence.

The identifier label may take many forms. Depending on the method of detection of the modified primers, there may be an identifying label or no label. With separation methods, by having modified primers, which can be detected by differential mobility, e.g. chromatography, or electrophoresis, one may be able to detect the modified primer solely by its mobility. Identifiers of this type may be referred to as mobility tags. Such modified primers may also be separable based on differential mass to charge ratios, e.g. by mass spectrometry. For the same and other techniques, one may require a label, which provides for detection by electromagnetic means, e.g. fluorescence or electron ionization. For example, the identifier may comprise a labeled nucleotide, which is capable of being joined to or included in a growing nucleic acid chain and has a label, which allows for differentiation, such as different fluorescers, electrophoretic tags, which allow for mobility discrimination,

electrochemical tags, chemiluminescent tags, gas chromatographic tags, etc. or physical separation, such as ligand-receptor combinations.

Similar labels may be bound to the primer, but fluorescers will usually be of limited diversity. Where the primer varies as to mobility, the diversity will be expanded by the number of different fluorescers, which one may detect and accurately distinguish. Usually, one does not wish to have more than about two different excitation sources, which greatly narrows the multiplicity achieved with fluorescent labels. However, fluorescent semiconductor nanoparticles, such as those described in *Science* (1998) *281*:2013-2016, may be of use as fluorescent labels with tunable, narrow emission bands with broad, matched excitation bands. Also, four-color fluorescent tag sets have been developed for DNA sequencing applications.

For mobility tags, one may use oligomers, such as peptides, oligonucleotides, organic oligomers, such as polyethers, polyesters, and polyamides, polyhaloalkanes, or substituents other than halo, such as cyano, oxy, thio, nitro, and the like. By virtue of the subject invention, using combinations of differentiation characteristics, a very large number of different attributes can be imparted to the primer and identifier, so as to permit a very large number of target sequences to be addressed in one or a few vessels.

In carrying out the method, the target species will be single stranded nucleic acid, DNA or RNA. The nucleic acid may come from any convenient source, prokaryotic or eukaryotic genomes, cDNA from prokaryotic and eukaryotic sources, mitochondrial DNA, rRNA, mRNA, synthetic DNA plasmids, cosmids, YACs, viruses, and the like. Where the DNA is double stranded, it will be denatured to provide single stranded DNA. The DNA may be further processed by mechanical fragmentation or restriction enzyme digestion. Conveniently, the fragments may be less than about 1 centiMorgan, usually less than about 10^5 nucleotides. The target nucleic acid is combined with the primer oligonucleotide under nucleic acid modification conditions, usually extension or restriction conditions.

The method employs as a modifying reagent system, besides the primers for each target nucleic acid, an enzyme having polymerase activity, which may also have 5'-3' nuclease activity, e.g. Klenow fragment of DNA polymerase, DNA polymerase 1, Taq polymerase, etc., an enzyme having 5' nuclease activity such as Cleavase®, ligase, restriction

endonuclease, nuclease or transcriptase activity. Accordingly, for primer modification the modifying reagent system may involve amplification, sequencing, mini-sequencing, SNP determination, strand cleavage, ligation, restriction, transcription or other purpose, which involves an interest in characterizing a plurality of target nucleic acid sequences. Usually, there will also be amplification of the target nucleic acid, performed in accordance with conventional methods, adding dNTPs and thermal cycling, as required, whereby the primer is extended. The thermal cycling involves a lower temperature step of extending the primer and a higher temperature step of denaturing the resulting duplex, followed by cooling to allow for hybridization of unextended primer to target in preparation for another step of primer extension. For methods of performing nucleic acid extensions using a polymerase, see, for example, *PCR Methods and Applications* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1994); Pastinen, *Clin. Chem.* (1996) *42*:1391; U.S. Patent No.'s 4,683,195, 4,683,202, 4,800,159, 4,965,188 and 5,008,182.

The particular method for performing the nucleic acid modification is not critical to this invention and any of a variety of ways may be employed, which may additionally involve various agents associated with the characteristic of interest, such as labeled terminators, labeled dNTPs, labeled ddNTPs, Cleavase®, ligases, nickases, restriction endonucleases, RNA, etc. See, for example, U.S. Patent No. 5,422,253 and U.S. Patent No. 5,712,124.

For sequencing and SNP determinations, as well as other assays where one wishes to know the particular nucleotide which has been added, either a labeled terminating nucleotide is employed or a labeled primer is employed, but in the latter case, four reaction vessels are used, each with a different terminator associated with a specific fluorescent labeled primer. Thus, each of the four terminating nucleotides would be associated with a different label, which would allow for their differentiation. Conveniently, fluorescent labels are used, such as FAM, ROX, TAMRA, TET, JOE and the like, or the "BIG" fluorescers are used, where fluorescein is bonded to another fluorescer, such as FAM, ROX, TAMRA, TET, JOE and other rhodamine or dichlororhodamine derivatives, and the resulting compounds are referred to as single energy transfer molecules. Another family of energy transfer dye sets incorporates cyanine as the primary energy donor. Dyes which may be used are described in U.S. Patent nos. 4,997,928 and 4,855,225 and PCT application nos. US90/06608 and

US90/05565. The terminator may be any molecule that is recognized by the polymerase being used, specifically binds to the complementary nucleotide present in the target, and cannot be extended. Thus, various modified or capped nucleotide analogs may be employed, but to the extent that the dideoxynucleotides are readily available, come with a variety of labels, and the conditions of their use are well established, they are the terminators of choice.

For sequencing, one would have a plurality of primer oligonucleotides. The targets to sequence could derive from one contiguous strand that could be 1 kb or more bases in length. Depending on the fidelity of the system and the capabilities of the assay method, the primers would be spaced at about 0.5 kb apart, desirably about 0.8 kb apart and even further, if the system permits accurate resolution at such spacing.

Alternatively, the targets for sequencing could derive from a plurality of strand fragments, plasmids or vectors. The reaction may be performed on the plurality of targets simultaneously, or in separate vessels according to the needs for associating distinct capture sequences with the different targets. For example, if each of the targets is contained in a unique plasmid then unique primer oligonucleotides can be employed in the same vessel. However, where the same plasmid vector or sets of vectors are employed for many constructs, the enzymatic reactions are conveniently performed using each plasmid or set of plasmids in a separate vessel in order to attach a unique capture sequence to each product. Separate vessels may also be employed in sequencing reactions where labeled primers are desired rather than dye-labeled terminators. The subject invention allows one to manipulate, separate or combine for analysis the different products from the different sets.

For genotyping single base positions to identify SNPs, triplet deletions or insertions using single base primer extension methods using a plurality of primer oligonucleotides, the targets may be present on a contiguous nucleic acid strand, or derive from a plurality of strands, strand fragments, chromosomes, plasmids or genomes, etc. Moreover, to enhance the specificity of the genotype determination at each position, both the sense and antisense strands present in the sample may be interrogated by primer oligonucleotides targeting each strand.

An exemplification of a primer reagent with identifiers that act as mobility tags for multiplexed genotype determination is shown in Figure 1. The genotype is determined by modifying the primer oligonucleotides in a single base extension reaction. The primers

hybridize to the homologous target sequence adjacent to the position of interest. In the presence of a polymerase enzyme and terminating derivatives of at least one of the four nucleotides, the enzyme adds to the end of the primer the base complementary to the base found in the next position on the target. The new base at the terminus of the primer cannot be extended, thus each primer increases in length by one base.

The primer reagent is comprised of a plurality of primer oligonucleotides that are divided into sets, and within each set the primer oligonucleotides have the same primer second part sequence (capture sequence) homologous to a capture oligonucleotide. Associated with each primer first part sequence is an identifier, which only need be unique within each set. The same identifiers may be used in the different sets. The identifier may be comprised of a sequence of n units, n being at least 1, and usually not more than about 20, but can be as many as 50 units. For convenience, the unit will be a nucleotide base, which can be incorporated into the primer oligonucleotides by standard automated DNA synthetic techniques. The primer first part sequences are conveniently designed to be of equal length, so that the overall length of the oligonucleotides, and therefore the mobilities, are differentiated by the length of the identifiers.

The length of the identifier is primarily chosen for convenience in the preparation of the primer oligonucleotides and for the separation and detection of the released primers. Each identifier may differ in length by at least one base, because single base resolution is normally achieved by common mobility-based assay methods, i.e. electrophoresis. The identifiers may differ by two base units to facilitate detection and distinguishing between modified and unmodified primers.

The number of identifiers determines the number of primer oligonucleotides within each set. In Figure 1, x represents the number of such identifiers within a set. The number of sets, multiplied by the number of identifiers in a set is the total number of multiplexed determinations.

For greater specificity in the genotype determination, the same type of analysis may be performed on both strands of a double stranded target. Thus, both the base and its complement are determined for each position of interest. There are many ways to design primer oligonucleotides for the analysis. For example, each set of primer oligonucleotides (cf.

Figure 1) may be dedicated to one strand of the target, or each set may contain the probes for analyzing both strands for given group of positions of interest.

Once the primer modification has been performed, the modified primer oligonucleotides may then be harvested. As previously indicated, the amplification will normally depend upon extending the primer with a polymerase, separating the extended primer from the target sequence, which normally involves thermal denaturation, recreating hybridization conditions, where unextended primer will hybridize to available sites on the target, and repeating the extension. This process may be repeated a sufficient number of times to provide the desired amount of extended primer. Depending on the nature of the extension reaction, duplexes may have to be denatured to provide the single stranded extended primers.

One may wish to separate modified primers from unmodified primers and other DNA present in the mixture. While this will normally not be necessary, such separation can be achieved for example, where the primer is modified by extension, by having a binding-member-labeled terminator, where the binding member has a complementary binding member bound to a support and is capable of ready release. By combining the reaction mixture with the support, only extended primers terminated with the binding member labeled terminator will be captured, and unbound and non-specifically bound DNA washed away. The captured extended primers may then be released. This secondary binding and release process may occur prior to or after the multiplexed strand capture and release process, preferably prior. While there are many different specific binding pairs that may be used, particularly convenient is the use of desthiobiotin and streptavidin, with biotin addition causing release by displacement. Other ligand-receptor pairs include:digoxin-antidigoxin; fluorescein-antifluorescein; saccharides and lectins, substrates/inhibitors and enzymes; etc.

Conversely, where the primer is modified by restriction a binding-member label may be used to separate the modified and unmodified primer oligonucleotides. By locating the binding-member label in the portion of the primer cut away from the portion containing the capture sequence and, if present, the identifier, then unmodified primer as well as the modification reaction side product can be separated away from the reaction product. The specific binding pairs described above may be used.

The number of different capture sequences usually will be at least about 3, more usually at least about 5, and may be one hundred or more, usually not more than about 50. In each group of capture sequences, there will usually be at least about 5 members, frequently at least about 10 members and there may be 1,000 or more. The number of capture sequences is related to the complexity of the sample and the number of different extended primers that may be distinguished. For example, if one is doing sequencing of a large DNA sample and one can distinguish 500 different extended sequences reliably, then the number of primers would be about the total number of nucleotides to be sequenced divided by 500. If 10 kb is to be sequenced, one would use about 20 primers, each primer having a different capture sequence and each primer would be associated with about 500 different extended sequences. In the case of SNPs, if one were interested in 5,000 SNPs and one had 100 distinguishable identifiers, one would have 5,000 primers (assuming that there was a single SNP at each site), 50 capture sequences, with each capture sequence associated with 100 different identifiers. Or, using the same number of identifiers and capture sequences one could analyze both strands in determining 2,500 SNPs. Thus the number of distinguishable tags determines the size of each group, which is analyzed, and the number of different capture sequences in the primer is determined by the complexity of the target divided by the group size. The number of modified primers will be determined by how many different events can be associated with a specific primer, varying from 1 in the case of SNPs to 500 or more in the case of sequencing.

The reaction mixture, either processed, or without any processing, is combined with the capture oligonucleotide under hybridization conditions. Usually, stringent conditions will be used, the degree of stringency depending upon the multiplicity of sequences, the length of the sequences, the T_m of the sequences, etc. Stringency may be achieved by variation in salt (buffer) concentrations, solvents, temperature and the like. The choice of stringency will be determined by the ability to specifically distinguish between the individual primers present in the extended primer mixture. Generally, the density of capture oligonucleotides bound on the support will be in the range of 10^5 to 10^{15} per mm^2, more typically about 10^8 to 10^{12} per mm^2, depending on the type of support, the desired concentration, the number of different sequences to be determined, and the like, where the ratio of capture sequences to primer sequences will be at least about 2:1 and preferably at least about 5:1, usually not exceeding about 10^3: 1.

Various wash and reaction buffers may be employed for reactions and washes. Buffers include saline, phosphate, carbonate, HEPES, MOS, Tris, TE etc. Generally the buffer concentrations will be in the range of about 1 to 500mM, more usually in the range of about 5 to 200mM. The use of the individual buffers is conventional and a particular buffer will be used in accordance with the particular application. In some cases, wash buffers will contain a minimal salt concentration or no salt whatsoever. Low salt wash solutions are particularly desirable just prior to release of the captured primer for use in, for example electrokinetic transport, such as electrokinetic injection for capillary electrophoresis applications.

After a reaction, a portion of the primer oligonucleotides will have been converted to a mixture of modified primer products. The primers and support(s) will be combined and incubated for sufficient time for hybridization between the primers and the capture oligonucleotides under the appropriate stringency conditions. The support(s) may then be sequestered from the liquid medium, using physical separation, centrifugation, filtration, magnetic separation, etc. and the support(s) washed with an appropriate buffer. The sequestering of the support from the liquid medium may take the form of flowing a buffer into the support area while forcing the original liquid out through an exit. The support(s) freed of non-specifically bound DNA, excess salts, templates, target, monomers, enzymes etc. will then be combined with an appropriate buffer, for beads redispersed in an appropriate buffer, in preparation for release of the captured primers.

The stringency conditions for release will be selected to provide a high degree of specificity for the displacement. The stringency of the conditions will depend to a degree on the nature and length of the displacement initiation sequences, the length of the capture sequences, the T_m of these sequences, the variation in primer sequences, and the like. Generally, salt concentrations for release will be in the range of about 0.1 mM to 100 mM, temperatures will be in the range of about 20 to 90°C, more usually 25 to 60°C, and while solvents other than water may be present, they will usually not be required, and if present, will generally be present in less than about 20% by volume. Obviously, the different parameters will be chosen to obtain the desired discrimination for strand release. Typically, at least one strand displacement oligonucleotide at a time will be combined with the support to cause the release. The ratio of strand displacement oligonucleotides to the anticipated maximum

number of captured primers will be at least 1:1, usually at least 5:1 and may be 10:1 or more, depending on cost, selectivity and time for carrying out the displacement.

In carrying out the differential strand release, at least one strand displacement oligonucleotide will be added to the support(s) comprising the captured modified primers. Incubation will be performed for sufficient time for a substantial proportion of the modified primers having the sequence of the displacing strand to be displaced. Once the modified primers have been released, they may then be harvested and processed. One may continue combining at least one strand displacement oligonucleotide with the support(s) comprising the remainder of the captured extended primers in a similar manner to induce the displacement of up to the rest of the extended primers. Preferably, the strand displacement oligonucleotides will be added consecutively, one at a time to the support(s) to provide for the release of the primers.

The release of the extended primers may take the form of having beads in a well having a membrane bottom and incubating the displacement strand with the beads for sufficient time for the appropriate group of extended primers to be released. Where the container has a permeable base, after sufficient time for displacement to occur, a differential pressure is created across the permeable membrane, so that the liquid containing the released extended primers is separated from the beads. Alternatively, the force to drive the liquid through the membrane may be generated by centrifugation. The liquid is isolated and then used for the next stage. For each displacement, the process is repeated, until all of the desired different extended primers have been substantially released. The beads may be washed between the additions of the different displacement strands. Alternatively, one could use a column in which beads are packed in substantially the same way as the container with the permeable membrane bottom. Another way is to incubate the displacement strand with the beads with agitation, centrifuge and then draw off the supernatant. Or, if the beads contain a magnetic core, they may be pelleted by application of a magnetic field to aid in drawing off the supernatant liquid. Again the process is repeated with each desired successive strand displacement. Instead of beads one could have a capillary with the capture sequences conjugated to the wall of the capillary. Alternatively, one could have a flow channel in a planar substrate with the capture sequence conjugated to a surface of the channel, to beads

contained within a section of a channel, or to a support confined within a channel. With a capillary or flow channel one could add the strand displacement oligonucleotide to the flow path. Other supports also find use, as appropriate.

Depending on the nature of the released extended primers, they may be isolated and processed in a variety of ways. Where the mobilities of the different extended primers are different, one may separate the extended primers by the different mobilities, depending on a detectable label for detection, when required. Separation can be achieved by electrophoresis, chromatography, gas or liquid, mass spectrometry, or the like. For electrophoresis and chromatography, a fluorescent tag can be provided on the primer or the terminator for detection of the individual bands of modified primers. Conventional conditions are employed for the separations. See recent reviews, for example, *Mol. Pathol.* (1999) *52*:117-124.

The subject method is applicable where large numbers of determinations are of interest. These include sequencing of nucleic acids, detection of SNPs, identification of nucleic acid fragments, and the like. The number of individual characteristics of interest will be at least about 10, more usually at least about 50, generally at least about 500, and may be 10,000 or more. For the most part, one vessel will be used, but in some instances, one may divide the determination into 4 vessels, one for each terminating nucleotide, or a multitude of vessels where similar cloning vectors may be uniquely addressed, so that a physical separation will contribute to the multiplexed diversity. In sequencing, for example, one could add the same family of primers to each vessel, but a different terminating nucleotide and carry out the extension in the presence of all four dNTPs, where each terminating nucleotide is differently labeled. After completion of the extension, one could combine the product from the four vessels and read the sequence by the mobility of the different extended primers, detecting the terminating nucleotide by the different labels. Alternatively, one could have the extension reaction carried out in a single vessel with all four terminating nucleotides present.

The number of different modified primers released at each stage will usually be at least 4, more usually at least about 15, and may be depending on the nature of the determination, at least about 100, and can be at least about 1,000 or more, usually being not more than about 500. The number of extended primers which are simultaneously released will depend on the

multiplicity required, the method of detection, the sensitivity and reliability with which different extended primers can be detected, available equipment, and the like.

For detection of SNPs, the use of strand displacement of the extended primers allows for carrying out the detection of a large number of SNPs, frequently 50 or more, where one is assaying for 100 or more, usually 250 or more, and up to 10,000 or more, in a single reaction vessel for the extension. One can then use the sequential release to partition the assay for a substantially reduced number of SNPs. By using the combination of independent release as one variable in conjunction with differential identifiers as a second variable, the number of permutations and combinations is greatly expanded. To illustrate, if one has 50 different identifiers, then sets of these 50 identifiers can be multiplexed by the number of different capture sequences. Thus, using 100 different capture sequences, one generates 5000 different detectable events. This allows for the use of identifiers, which may have limited diversity, such as fluorescers, gas chromatographic labels, etc.

The subject invention greatly simplifies carrying out highly multiplexed reactions in one or a few vessels, and greatly simplifies highly multiplexed analyses of many reactions. By having two different variables, the capture oligonucleotide and an identifier, greater flexibility is obtained in the choice of the identifier and one can provide for sharper differentiation in the detection of the different identifiers. Also, the number of different molecules required to be synthesized is reduced, since one may employ a smaller repertoire of identifiers, while still achieving the required diversity for the identification of the individual events. The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1

The strand displacement method was demonstrated using a two-step process, comprising hybridization of a fluorescent oligonucleotide to a capture oligonucleotide surface-bound to a bead, followed by displacement of the fluorescent oligonucleotide into solution due to the addition of a strand displacement oligonucleotide.

Hybridization

Materials:

Streptavidin-coated magnetic beads (Seradyn, 1 μm diameter)

TENSS Hybridization Buffer (0.1 M Tris. p 8.2, 0.3 M NaCl, 25 mM EDTA, 0.1% BSA, 0.1% T-500 dextran, 40 μg/ml salmon sperm DNA)

Primer oligonucleotide: SEQ ID 1

Capture oligonucleotide: SEQ ID 2

Microplate fluorimeter *fmax* (Molecular Devices)

Black, C-bottom plates "Microfluor" (Dynatech)

Method:

Equimolar amounts of the primer and capture oligonucleotides (SEQ ID 1 and SEQ ID 2) were combined in TENSS buffer. The solution was incubated at 70°C for 10 min to remove secondary structure, and left at room temperature for 1 h to allow for annealing of the oligos. Streptavidin-coated magnetic beads were added to the solution and the mixture was tumbled at room temperature for 1 h. A 5 μl aliquot was removed and the fluorescence measured to determine the background fluorescence intensity. Strand displacement was then performed.

Strand Displacement

Material:

Low salt buffer LOSST (0.1 M Tris pH 8.2, 0.01 M NaCl, 0.025 M EDTA, 0.1% BSA, 0.1% T-500 dextran, 100 μg/ml salmon sperm DNA)

Strand displacement oligonucleotide: SEQ ID 3

Method:

Aliquots of the mixture containing 5 to 20 μg of beads were washed with 20μl cold LOSST buffer. Immediately thereafter 20 μl of LOSST buffer containing 340 pmol (A_{260} = 0.1) of strand displacement oligonucleotide was added, the mixture was vortexed briefly, and incubated at 37°C for 30 min. A 5 μl aliquot was removed and the fluorescence intensity was measured. Table 1 shows the results of adding the displacer oligonucleotide (SEQ ID 3) to

varying amounts of beads with the bound duplex. The increase of the fluorescence in the supernatant indicates that the primer oligonucleotide has been released from the primer/capture duplex bound to the bead.

Table 1.

Amount of beads [μg]	Increase in fluorescence of supernatant [rfu]
5	2.81
10	4.68
15	6.56
20	8.43

Example 2

The same two-step process of Example 1 was employed for examing the effect of changes in temperature during displacement.

Hybridization

Same as Example 1.

Strand Displacement

Method:

An aliquot containing 20 μg of the bead-hybrid duplex was washed with 20 μl cold LOSST buffer. Immediately thereafter, 60 μl of cold LOSST buffer containing 34 pmol (A_{260} = 0.01) of strand displacement oligonucleotide was added, the bead solution was mixed by pipetting, and incubated at the specified temperature. At the indicated time intervals a 20 μl aliquot was removed, the beads pelleted, and a 5 μl sample removed for fluorescence analysis. The results are shown in Table 2. The data show increased fluorescence due to release of the primer from the primer/capture duplex bound to the bead.

Table 2.

Time [min]	Increase in fluorescence of the supernatant [rfu] vs. T [°C]	
	4°C	21°C
1	0.125	0.262
2	0.131	0.410
5	0.275	0.558

Example 3

Multiplexed strand displacement with sequential and simultaneous release was demonstrated using four PCR amplicons, comprising hybridization of the amplicons to a set of capture oligonucleotides surface-bound in a defined region of a microfluidic channel, followed by sequential displacement of each amplicon into solution due to the addition of the appropriate strand displacement oligonucleotide.

A microfluidic device was fabricated in a planar, polymethyl methacrylate substrate. Briefly, a 120 μm wide by 50 μm deep channel was formed, connecting two reservoirs, each with a 5 μL capacity. After treating a region of the channel with surface immobilization reagents as described below, the channel was covered by thermally bonding an acrylic film to complete the device fabrication.

First, a biotin-coated surface was prepared using a benzophenone-albumin-biotin (BAB) conjugate. The BAB conjugate was prepared by the reaction of an anhydrous dimethylformamide solution of 10 mg/mL each of biotin-X-X-NHS (Sigma) and 4-benzoylbenzoic acid with a 17 mg/mL solution of bovine serum albumin (BSA) in PBS. The product was purified by dialysis against water. The solution was protected from room light during the reaction and dialysis. A 10 mg/mL solution of BAB in 1X PBS buffer was introduced into the channel, and irradiated for 20 min through a mask defining a 1.5 cm length of the channel using a collimated 100W mercury lamp. Surface immobilization of BAB to the PMMA substrate occurred via photoaddition of benzophenone to the polymer. Following the

reaction the channel was rinsed first with 0.05% aqueous Triton X-100 and then with distilled water.

A streptavidin-coated surface was prepared by incubating the biotin-coated surface with 0.1 mg/mL streptavidin in TE buffer for 30 minutes. The channel was rinsed with aqueous 0.05% Triton X-100 and then distilled water.

PCR reactions were performed with four primer pairs (see Table 1) for the amplification of four target sequences: beta actin, dystrophin exon 3, dystrophin exon 13, and dystrophin exon 47. For each target, one PCR primer, referred to as the primer oligonucleotide, was designed to bind to the immobilized capture oligonucleotide. The amplicons were labeled with fluorescent tags by including R110-dUTP and R110-dCTP monomers in the dNTP mix. The three dystrophin targets were amplified using the following temperature profile: 94°C for 5 min, and 30 cycles of 94°C for 2 min, 56°C for 1 min, 65°C for 4 min. The beta actin target was amplified using the temperature profile: 94°C for 2 min, and 30 cycles of 94°C for 1 min, 56°C for 30 sec, 72°C for 30 sec, and an extension of 72°C for 5 min. Following amplification the products were separated by agarose gel electrophoresis, isolated using a gel extraction kit (Qiagen) with elution in water.

Capture oligonucleotides were prepared for each amplicon, each with a biotin moiety at the 5' end (see Table 3). Each capture oligonucleotide was added to the appropriate amplicon in two-fold molar excess, heated to 95°C for 10 min and then left to cool at room temperature for 1 h. These solutions were combined, and injected into the channel and incubated for 10 minutes to allow the formation of multiplexed, capture oligonucleotide/primer-amplicon complexes bound to the surface via biotin-streptavidin adduct formation. The channel was then washed with 1 mM $MgCl_2$, 50 mM Tris (pH 8.0), 0.01% Tween 20, and a final wash of 1 mM $MgCl_2$, 50 mM Tris (pH 8.0).

The captured primer oligonucleotides (i.e. primer-amplicon) were released both simultaneously and sequentially in separate experiments. The procedure was the same except for the number of strand displacement oligonucleotides included in the release buffer. To the upstream reservoir was added 1 mM $MgCl_2$, 50 mM Tris (pH 8.0), and 5 uM strand displacement oligonucleotide(s) (see Table 3). The downstream reservoir contained a CXR

fluorescent ladder (Promega) of 60 – 400 bp in the same buffer. Electrodes were contacted with the two reservoirs, and the displacement solution was electrokinetically moved over the immobilization region and into the downstream reservoir. Any displaced primer-amplicons joined the pool containing the sizing ladder. When the strand displacement oligonucleotide was added singly, the channel was washed with 1 mM $MgCl_2$, 50 mM Tris (pH 8.0) and the buffer in the reservoirs was changed between each release. The order of introduction of the strand displacement oligonucleotides was SEQ ID 14, 10, 18 and then 6.

This solution was then analyzed by capillary electrophoresis in a microchannel in a polymethyl methacrylate substrate as described in U.S. Patent No. 5,570,015. In the electropherogram, peaks for the primer-amplicon were observed at the expected time, as confirmed by the sizing ladder. The electropherogram for the sample with simultaneous release is shown in Figure 2, and those for the sequential samples with single release are shown in Figure 3.

Table 3

Target	beta actin	dystrophin, exon 3	dystrophin, exon 13	dystrophin, exon 47
PCR primer	SEQ ID 4	SEQ ID 7	SEQ ID 11	SEQ ID 15
Primer oligo	SEQ ID 5	SEQ ID 8	SEQ ID 12	SEQ ID 16
Capture oligo	SEQ ID 2	SEQ ID 9	SEQ ID 13	SEQ ID 17
Strand Displ. oligo	SEQ ID 6	SEQ ID 10	SEQ ID 14	SEQ ID 18
Amplicon length	290	410	238	170
Effective length	310	430	260	190

Example 4

Multiplexed strand displacement of the products of a cycle sequencing reaction of a 290 bp region of the beta actin gene was demonstrated, and verified by sequencing the displaced primers/extended primers in a commercial DNA sequencer. Two types of capture oligonucleotide structures were also compared.

The microfluidic device, BAB preparation and immobilization, and avidin treatment were as described in Example 3. Note that streptavidin was replaced with avidin in this experiment.

A cycle sequencing reaction of a 290 bp region of the beta actin gene was performed using primer oligonucleotide SEQ ID 19 as the sequencing primer, and a PCR product as the template. The reaction was carried out using 9.6 pmol of sequencing primer and 40% by volume of Big Dye Ready Mix according to standard protocols. After the cycling reaction, an 8.5 μL aliquot was combined with 0.75 μL 4 M NaCl and 0.75 μL of 5 μM capture oligonucleotide. Two capture oligonucleotides were used, SEQ ID 20, which has a spacer moiety between the capture sequence and the displacement initiation sequence, and 21, which has a spacer moiety between the biotin and the capture sequence. The solutions were heated to 95°C for 5 min and cooled at room temperature for 1 hr. The capture oligonucleotide/cycle sequenced product complex was captured in the channel as described in Example 3.

Release of the cycle sequencing products was done by heating 1X TE solution to 95°C, passing the solution through the channel and collecting the solution.

The solution was analyzed for the cycle sequencing products on an ABI 310 DNA sequencer, in a 47 cm long, 50 μm capillary with POP-6 matrix according to the standard operating procedure. The full length of sequencing products was obtained when using SEQ ID 20 as the capture oligonucleotide.

It is evident from the above results that the subject method provides for great versatility in scoring a large number of events in a reliable and accurate manner. By using a combination of varying oligonucleotides which may be individually released with a repertoire of identifiers, one is able to multiply the number of identifiers with the number of varying oligonucleotides, greatly enhancing the number of individual events which may be scored, while still permitting a simple analytical method. Since the number of molecules, which must be assayed in one determination, is only a fraction of the total number of events to be determined, one can provide for substantial distinctions between the identifiers, enhancing reliability and accuracy in scoring the events.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporate by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.